CHAPTER V

DISCUSSION

In the present study, the experiments were conducted to investigate the effect of serotonin depletion on the dural inflammation-induced phosphorylation of NR1 receptor, NR1 receptor expression, and trigeminal nociception. From the results, it could be discussed as the following.

Effect of dural stimulation on trigeminal nociception in normal rats

In our experiment, two models of dural stimulation were employed. The results demonstrated that topical application of inflammatory soup (IS) containing a mixture of inflammatory mediators (histamine, serotonin, bradykinin, each at 1 mM, and 0.1 mM prostaglandin E_2, pH 5.5) on exposed dural surface for 30 minutes can activate trigeminal nociceptive pathway indicated by the increase in the number of Fos-immunoreactive (-ir) cells in trigeminal nucleus caudalis (TNC). However, low-pH phosphate-buffered artificial cerebrospinal fluid (low-pH CSF; pH 4.7) applied on dura cannot activate trigeminal nociception. Although, many studies showed that dural application of low-pH CSF can sensitize peripheral nociceptors that innervate dura and trigeminal brainstem neurons [11, 13], low-pH CSF itself was not potent enough to induce Fos expression in dorsal horn neurons of the spinal cord in our study.

The rationale for using bradykinin, histamine, serotonin, and PGE_2 was that: 1) these agents are found endogenously, are believed to be released locally during inflammation, and are thought to be released in the vicinity of the dural sinuses by increased plasma extravasation, and mast cell degranulation induced by neurogenic inflammation; 2) these agents can activate and sensitize somatic and visceral nociceptive primary afferent neurons; 3) these agents are potent algesics in humans; and 4) although all three inflammatory mediators alone exert excitatory and sensitizing effects on nociceptors, some studies have shown a synergism between the excitatory

actions of inflammatory mediators and low pH to induce sensitization of nociceptor [13, 116]. In most recent studies, the mixture of inflammatory mediators at low pH (IS) was used because it mimics the neurogenic inflammation occurring during a migraine attack and it is most effective at inducing hypersensitivity in peripheral nociceptors and central neurons [13, 55, 117].

Fos expression in TNC induced by dural application of low-pH CSF has never been demonstrated before. In contrast, The study by Malick and coworkers [118] revealed that dura stimulated with IS followed by brief mechanical indentations could induce Fos expression in dorsal horn neurons of spinal cord compared to sham operation. Our study showed that application of IS alone, but for a long period (30 minutes), can also induce Fos expression in dorsal horn neurons. In our experiment, The Fos-ir cells was mainly distributed in the ventrolateral part and extended across dorsomedial part of the dorsal horn which is the similar pattern with the result from Malick's study. The Fos distribution in this area was a character of Fos expression induced by dural stimulation, not by facial (supraorbital skin) stimulation that demonstrated Fos expression restricted in ventrolateral region [119].

Although, the *c-fos* is an early-response proto-oncogene and is rapidly and transiently expressed in response to noxious inputs, Fos expression cannot be detected at 30 minutes after dural stimulation in our study. In most studies, Fos expression was detected at least 1 hour after stimulation. In control group, there was a substantial increase in Fos expression. This increase was due to neuron irritation, which was difficult to avoid, during drilling a large portion of parietal bone.

Effect of dural stimulation on NR1 receptor expression in normal rats

In our study, using immunohistochemical technique, NMDA receptor NR1 subunit expression did not change following meningeal inflammation on the ipsilateral and the contralateral sides at both 30 minutes and 2 hours after stimulation. There were many studies of NR1 expression in various model of inflammation. The lack of change in NR1 subunits is consistent with the findings of Caudle *et al.* [83], Zou *et al.* [73, 120], Brenner *et al.* [2], and Gaunitz *et al.* [121].

Caudle *et al.*, using western blot, demonstrated no change in the levels of NR1 in the lumbar region of spinal cord (L_2-L_5) on the ipsilateral side following the inflammation induced by carrageenan at all time points (2, 6, and 24 hours). In 2000, Zou *et al.*, using immunofluorescence, revealed that there was no significant difference in the number of the NR1-like immunoreactive neurons in lamina I-VII in the lumbosacral segments (L_4-S_1) on the ipsilateral and the contralateral sides at 30 minutes after capsaicin or vehicle injection. They also showed that there was no significant change in the immunoblots for NR1 subunits after capsaicin injection. Brenner *et al.*, using western blot analysis, could not detect the change in NR1 expression in the lumbar segments (L_4-L_5) on the ipsilateral side at 30 minutes following noxious heat stimulation of hind paw. It can be concluded from these finding that NR1 protein expression is not influenced by peripheral inflammation.

In an excitotoxity induced spinal cord injury model, Caudle and colleagues [122] found that, in contrast to the short term peripheral nociception models, NR1 protein was up regulated in the spinal cord following the excitotoxic injury. These data suggest that sustained injuries produce not only changes in NMDA receptor phosphorylation, but also changes in protein expression. The changes in NMDA receptor protein could lead to a more extended period of central sensitization than the readily reversed NMDA receptor phosphorylation. In chronic constriction injury model, Wilson and coworkers [123] demonstrated that NR1 protein was down regulated in the spinal cord following the constriction of sciatic nerve. In these injury models, NR1 expression was studied at more than 10 days following injury induction, which was a long period compared to peripheral inflammation model (up to 24 hours). Thus, it may suggest that a long period of time is required for the induction of new NR1 protein expression in spinal cord.

According to the result from our study, which demonstrated no relation between the trigeminal nociception and the expression of NMDA receptor, together with the result from peripheral inflammation model, it can be imply that the up-regulation of NMDA receptor is not a mechanism of pain hypersensitivity during a short period of peripheral inflammation.

Effect of dural stimulation on NR1 receptor phosphorylation in normal rats

In the present study, using immunohistochemical technique, the phosphorylation of NR1 subunit of NMDA receptor at serine-896 was mainly demonstrated in superficial dorsal horn of TNC on the ipsilateral side following meningeal inflammation. This phosphorylation was rapidly induced (30 minutes) and persisted for long period (up to 2 hours). The relation between phosphorylation of NR1 subunit and inflammation were reported in several studies

Zuo and coworkers [82] reported in 2004 that NR1 was phosphorylated at serine-896 after capsaicin injection and that the phosphorylation of NR1 observed in their study was attenuated by the pretreatment with PKC inhibitor. Brenner and colleagues [2] demonstrated that brief noxious heat (1 minute) applied to the hind paw of rats produces an increase in phosphorylation of NR1 at a PKC-dependent site, serine-896, in the superficial dorsal horn neurons. Similar to our study, They could not detect phosphorylation of NR1 serine-896 in naïve rats. The induction of NR1 phosphorylation at serine-896 was a rapid response (< 2 minutes) following a noxious heat stimuli but not innocuous heat stimulus. The number of pNR1-immunoreactive neuronal profiles in the superficial dorsal horn is highest 30 minutes after noxious heat stimulation and persists for up to 1 hour. In their study, phosphorylation of NR1 was resolved quickly, unlike our study which still showed an intense phosphorylation at 2 hours. The difference of the existence of phosphorylation of NR1 may due to the different duration of stimulus applied to the animal. In our experiment, the dura was stimulated for 30 minutes while in Brenner and colleague's study the noxious stimulus was applied only for 1 minute. Furthermore, the phosphorylation of NR1 on serine residues was recently reported in carrageenan inflammation model by Caudle and coworkers [83]. Their results showed that after injection of carrageenan into hind paw to induce the inflammation, the phosphorylation of NR1 on serine residues were observed in lumbar spinal cord within two hours and return to normal in 6 hours. They also showed that no phosphorylation on NR1 threonine or tyrosine residues was observed. The rapid and transient characteristic of the induction of phosphorylation of NR1 following a noxious stimulus or inflammation suggests that NR1 phosphorylation may represent an early step in the generation of central sensitization and pain hypersensitivity.

Previously, it was demonstrated that PKC phosphorylation at serine-896 of the NMDA receptor NR1 subunit suppresses ER retention, which result in forward receptor trafficking and then induce the insertion of NMDA receptor to synapse. These processes finally enhance glutamate current [3]. Brenner and coworkers [2] confirmed the evidence corroborating the ER as the site of NR1 phosphorylation. Modulation of synaptic glutamate current contributes to activity-dependent synaptic plasticity [124], and phosphorylation of the NMDA receptor is likely to play an important role in the regulation of glutamate currents [125]. These observations, in conjunction with the substantial evidence implicating spinal cord NMDA receptors in the generation and maintenance of central sensitization and pain hypersensitivity, suggest a role for NMDA receptor subunit phosphorylation in the central sensitization activated by peripheral nociceptive input.

Effect of serotonin depletion on trigeminal nociception induced by dural stimulation

Our study showed that serotonin depletion potentiated trigeminal nociception. The result from our study demonstrated that depletion of serotonin by PCPA treatment potentiated the trigeminal nociception in both control and dural inflammation-induced groups as indicated by the increase in the number of Fos-ir cell. Increased in trigeminal nociception in control group may implied that 5-HT depletion may facilitate the trigeminal nociception in general. Increased Fos immunoreactivity in serotonin-depleted rats was more evident in IS group, which indicated that the trigeminal nociception is further facilitated in response to dural inflammation in low serotonin condition. This result supports the study of Supornsilpchai *et al.* [111]. They demonstrated that the numbers of CSD-evoked Fos-ir cells in TNC were significantly greater in the low 5-HT group than those obtained from the normal 5-HT group. They suggested that 5-HT depletion enhanced CSD-induced trigeminal nociception by increasing sensitivity of trigeminal nociceptive system.

Effect of serotonin depletion on NR1 receptor expression

Our study showed that neither serotonin depletion itself nor serotonin depletion combined with dural stimulation produce change in NMDA receptor NR1 expression. The effect of low serotonin condition on NMDA receptor expression has never been demonstrated before. Previous reports in several inflammation studies demonstrated no change of NR1 expression at any time points (30 minutes up to 24 hours) was observed after application the stimulus (e.g. carrageenan injection, capsaicin injection, and noxious heat stimulation). Thus, it does not surprise that pretreatment with PCPA, which does not nociceptive or inflammatory stimulus, did not alter NR1 expression. As the results of no effect of serotonin depletion on the NR1 expression, we can imply that there are no relation between the expression of NMDA receptor and the facilitation of the trigeminal nociception observed in serotonin depleted state. However, serotonin was depleted for only 3 days in our study. Up-regulation of NR1 receptor may be observed if serotonin was depleted for longer period. Thus, whether increase in frequency of headache attacks observed in chronic daily headache patient involves the up-regulation of NMDA receptor or not has remained to be elucidated.

Effect of serotonin depletion on NR1 receptor phosphorylation induced by dural stimulation

In the present study, we demonstrated the increment of NR1 receptor phosphorylation in serotonin depleted state. Pretreatment with PCPA increased the number of pNR1-ir neurons in TNC of both control and dural inflammation-induced groups. Similar to Fos expression, the increase in NR1 phosphorylation in serotonin-depleted rats was more evident in IS group. This result demonstrated that the NR1 phosphorylation is further facilitated in response to dural inflammation in low serotonin condition.

It has been hypothesized that depletion of serotonin may predispose the patients to migraine attack, although the mechanisms are still unknown. In this study, we demonstrated that serotonin depletion altered the nociceptive processing in the spinal cord level. Phosphorylation of NMDA receptor which leads to the increased

number of this receptor at the synapse may involve in the nociceptive facilitation in serotonin depleted condition. Newly inserted NMDA receptors to synapse may reduce nociceptive threshold and make central neurons to be more excitable. In this study, we demonstrated that serotonin depletion promoted dural inflammation-induced phosphorylation of NMDA receptor at an early time point (30 minutes). Thus, this may suggest that hypo-serotoninergic state boosts the development of central sensitization. Our results also demonstrated the sustain level of NMDA receptor phosphorylation at 2 hours after dural stimulation. Thus, this may suggest that low serotonin condition not only boosts the development of central sensitization, but also maintains it.

The increase in NMDA receptor phosphorylation in serotonin-depleted state may result from higher input from sensory neurons innervating meningeal vessels. In the study of NMDA receptor phosphorylation, it was previously demonstrated that NMDA receptor was involved via a feed-forward mechanism in its own phosphorylation, since the phosphorylation of NR1 subunit is attenuated by intrathecal injection of the NMDA receptor antagonist, MK801 [2]. It may imply that greater activated peripheral nociceptors transmit more nociceptive input to central nociceptive neurons, resulting in more phosphorylation of NR1 receptor. It has been reported in serotonin depleted state that the cerebral microvessel dilatation induced by nitric oxide was higher than the response in control [103]. Thus, it can be implied that the response of dura vessels to the IS in our study was potentiated by the serotonin depleted state. Hyposerotoninergic state may also alter trigeminal nociceptive processing at peripheral and central terminal of primary trigeminal nerve fibers.

It was known that the descending 5-HT containing fibers arise from the nucleus located within the rostral ventromedial medulla (RVM). In the RVM, the nucleus raphe magnus (NRM) is the main source of serotonergic fibers in the spinal cord. The serotonergic fibers also descend to the spinal cord through the dorsolateral funiculus and bifurcate their axon collaterals to innervate dorsal horn neurons, in particular lamina I and IIo [126, 127]. There is an evidence for apposition of serotonergic fibers at central primary afferent terminals in the dorsal horn and trigeminal nucleus [97, 98]. A 5-HT_{1A} receptor agonist, 8-OH-DPAT markedly reduces the release of glutamate from the primary afferents, particularly C afferents [128, 129]. The subpopulation of primary afferent fibers also express 5-$HT_{1B/1D}$ [130]. The activation of those receptors expressed on primary afferent fibers is reported to produce an antinociceptive effect. Taken together, serotonin modulates nociceptive

input from primary afferent fiber by inhibiting glutamate release from presynaptic terminal via 5-HT_{1A} or 5-$HT_{1B/1D}$ receptors located on central terminal of primary afferent. In serotonin-depleted state, this modulation may be absent and primary neurons become more responsive to stimuli and enhance the glutamate release.

It was recently reported that THP-1, tryptophan hydroxylase – an enzyme synthesizing serotonin, was present in medium-sized trigeminal neurons of the major functional subtypes including those containing CGRP, a potent vasodilator present in sensory neurons innervating cerebral vessels, or IB4, a marker of nociceptors that are less likely to contain CGRP but may contain other peptides. In female mice, both TPH-1 mRNA and protein change across the menstrual cycle. The mRNA and protein were presented at higher levels at proestrus, the high estrogen phase of the estrous cycle, than at diestrus, the low estrogen phase of the cycle. This may imply that the serotonin fluctuation in trigeminal ganglia through estrous cycle contributes to menstrual migraine in human [131]. It may imply from our study that pretreatment with PCPA may directly reduce serotonin in trigeminal ganglia and alter trigeminal nociceptive processing at peripheral level. This may explain the result of the enhancement of IS induced NR1 phosphorylation in serotonin depleted rats. Sensory fiber innervating dura may increase response to IS and transmit more nociceptive information to central neuron, resulting in more evident NR1 phosphorylation observed in serotonin-depleted rats with dural stimulation by IS.

Relationship between NR1 receptor phosphorylation and trigeminal nociception

Our study demonstrated the relationship between NR1 phosphorylation in TNC and trigeminal nociception. There was a strong positive correlation between the number of pNR1-ir cells and the number of Fos-ir cells ($r^2 = 0.957$, $P < .001$). The correlation could also be presented by the linear regression of $y = 0.520x$. Because both pNR1- and Fos-ir cells were absent in naïve rats, the equation should pass the origin (0,0). Thus, we did not include a constant in the equation. This study is the first that demonstrates the correlation between NR1 phosphorylation and Fos expression (trigeminal nociception). There were several studies confirmed the association between NR1 serine phosphorylation and nociceptive behaviors.

Several line of evidence has revealed that the NR1 serine phosphorylation also associated with nociceptive behavior. In an excitotoxity induced spinal cord injury model, Caudle and coworkers [122] examined NR1 phosphorylation and found the association of an increase in NR1 serine phosphorylation and hyperalgesia and allodynia behaviors. In a hind paw inflammation with carrageenan model, Caudle and colleagues [83] also showed that NR1 serine phosphorylation, which peaked at 2 hours, paralleled thermal hyperalgesia following the carrageenan injections, which peaked in the range of 2 to 6 hours.

The strong correlation between NR1 receptor phosphorylation and nociception, in conjunction with association between NR1 receptor phosphorylation and nociceptive behaviors, suggests that NR1 receptor phosphorylation, in addition to Fos expression, may be used as an alternative marker to measure (indicate) the nociception. NR1 phosphorylation has more rapid induction profile (less than 2 minutes) than Fos (more than 30 minutes). Thus, the using of NR1 receptor phosphorylation as nociceptive indicator has an advantage over Fos expression, especially in an early stage of nociceptive induction.

In serotonin depleted rats, strong positive correlation between the number of pNR1-ir cells and the number of Fos-ir cells still presented ($r^2 = 0.941$, $P < .001$). However, the slope of the equation shifted to 0.706. Thus, the linear regression became $y = 0.706x$. This shifted slope of the equation indicated that serotonin depletion not only intensified chemically induced trigeminal nociception, but also increased sensitivity and vulnerability of trigeminal pathway, especially central neuron in TNC, to stimulus.

Descending serotonergic system may play an important role in this phenomenon. The binding sites for 8-OH-DPAT, an agonist specific to 5-HT_{1A}, are localized in the superficial dorsal horn (laminae I and II) [132]. There are behavioral studies showing that the stimulation of the raphe magnus causes a inhibition of nociceptive dorsal horn neurons that seems to be mediated by the activation of the descending 5-HT systems, since pretreatment of PCPA reduces the effect of raphe stimulation [133, 134]. The administration of 5-HT causes hyperpolarization (outward current) in about 50% of central neurons of lamina II. This inhibitory effects is mimicked by the 5-HT_{1A} agonist 8-OH-DPAT and blocked by the 5-HT_{1A} antagonist WAY100635 [135]. Taken together, it may imply that serotonin modulates nociceptive processing in central neurons via 5-HT_{1A} receptors. Low serotonin

condition may diminish this descending serotoninergic inhibition and enhance nociceptive sensitivity of dorsal horn neuron.

This may explain the phenomena in recent clinical experiments. The development of the migraine-like headache seems to parallel with the depletion of serotonin, as caused by the reserpine, with its lowest value attained 5–7 hours after administration [136]. Drummond [109] found that normal subjects that consumed an amino acid drink that omitted L-tryptophan (thereby reducing brain serotonin synthesis) boosted dizziness, nausea, and the illusion of movement to levels that approached those of migraineurs. Drummond [110] further investigated the sensitivity to light in migraine sufferers and control subjects after consumption of an amino acid drink which contained L-tryptophan (balanced amino acid condition) or of a drink that omitting L-tryptophan which produced a short-term reduction in brain serotonin synthesis (tryptophan depletion condition). Migraine sufferers reported more intense nausea, headache, glare- and light-induced pain than controls. In addition, glare- and light-induced pain was greater in the tryptophan depletion condition than in the balanced amino acid condition, in both migraine sufferers and controls. Eight hours after the amino acid drink, tryptophan depletion augmented headache in migraine sufferers and aggravated nausea in migraine sufferers and controls. These findings, together with our results, suggested that a reduction in brain synthesis of serotonin increased susceptibility of migraine attack in migraine sufferers.

Further study

We demonstrated that dural inflammation induced phosphorylation of NR1 subunit of NMDA receptor at serine-896 in superficial dorsal horn of TNC. Whether or not this phosphorylation is mediated by PKC remains to be determined. In order to substantiate the result, specific kinase inhibitors should be used in future study. In this study, we demonstrated that short term depletion of serotonin did not alter NMDA receptor expression. To demonstrate whether increase in frequency of headache attacks observed in chronic daily headache patient involves the up-regulation of NMDA receptor or not, chronic serotonin depletion model should be used in future study.